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PRINCIPAL INVESTIGATOR: Muling Mao
Bruce D. Cuevas, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
M.D. Anderson Cancer Center
Houston, Texas 77030

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6. AUTHOR(S)
Muling Mao
Bruce D. Cuevas, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas
M.D. Anderson Cancer Center
Houston, Texas 77030
E-Mail: mmao@mdanderson.org

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Tumor suppressor MMAC1/PTEN/TEP1 is considered related with breast cancer formation or progression due to a collective evidence of mutant MMAC1 in various advanced cancers, especially breast carcinoma. Our preliminary data as well as results of most recent study have provided further evidence of MMAC1 regulation on PI3K pathway signaling and cell activities, such as cell growth and survival. Our observation of differential modulation of MMAC1 on protein tyrosine phosphorylation in cells indicates a complex role of MMAC1 in multiple cell signals. Thus, the established MMAC1 transgenic mice model will undoubtedly help to determine the role of MMAC1 in mammary development and tumorigenesis *in vivo*.

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Introduction

MMAC1/PTEN/TEP1 is a tumor suppressor gene on 10q23, which is aberrant at the level of Loss of heterozygosity (LOH), mutation, methylation, protein stability and function in a significant portion of breast cancers (1-4). Germ line mutations in MMAC1 are responsible for the Cowden's breast cancer predisposition syndrome, validating MMAC1 as a critical target in breast tumorigenesis (5,6). As reported, about 30% of women from the Cowden disease families developed cancer, of which the great majority (78%) was of the breast. MMAC1 is also functionally inactivated in a high proportion of leukemia and lymphomas by methylation independent mechanisms (7). Therefore, MMAC1 is an important tumor suppressor gene, particularly in breast tissue.

LOH of MMAC1 is a frequent (>40%) event in invasive breast cancers (3,4). In support of the concept of functional inactivation of MMAC1 in breast cancer, 15% of invasive ductal breast cancers lack detectable MMAC1 protein and 18% have decreased MMAC1 protein levels with the majority of cases demonstrating LOH at 10q23. Inactivation of MMAC1 may represent a late event, as MMAC1 mutations occur at a higher frequency in advanced breast cancers and LOH at 10q23 is strongly correlated with increased tumor invasiveness and poor differentiation (3,4). The stability and enzyme activity of the MMAC1 protein are regulated by phosphorylation at serine, and, as we have demonstrated, tyrosine residues by growth factors and src tyrosine kinases (8,9). Thus, the function of MMAC1 and its down stream pathway may be altered in breast cancers by post-transcriptional modification as well as by genetic effects.

MMAC1 dephosphorylates the site on membrane phosphatidylinositols (PtdIns) phosphorylated by phosphatidylinositol 3 kinase (PI3K) (10,11). MMAC1 also dephosphorylates serine, threonine and tyrosine residues in proteins (12). Thus, MMAC1 has the ability to dephosphorylate proteins, lipids and inositols, suggesting that MMAC1 may alter the activity of multiple proteins as well as of the PtdIns pathway. Introduction of a wild-type MMAC1 gene into breast, prostate, or glioma cancer cell lines lacking functional MMAC1 protein decreases signaling through the PI3K pathway as indicated by a decrease in PtdIns3,4,5 P3 levels and alterations in downstream events including phosphorylation or localization of AKT, p70S6K, GSK3-α, GSK3-β, Bad, and 4E-BP1 (13-16).

The PI3K target, AKT is the cellular homologue of the v-AKT oncogene and has been widely shown to be involved in survival signals induced by various growth factors (17, 18). The pleckstrin

homology (PH) domain on AKT mediates high affinity interactions of AKT with the PI3K lipid product, PtdIns (3,5) P2 resulting in AKT recruitment to the cell membrane for activation (19). Full activation of AKT is at least partially dependent on phosphorylation of thr 308 and ser 473 by PDK1 and PDK2 respectively (20). Activated AKT is thought to mediate the activation of cell cycle mediators and inactivation of apoptotic proteins.

Body

Task #1 To develop and establish transgenic mouse models which display mammary gland specific expression of the wildtype and inactive forms of the MMAC1 tumor suppressor.

We have inserted the wild type (wt) MMAC1 gene into murine mammary tumor virus (MMTV) vector that specifically targets mammary tissue. An inactive form of MMAC1, which has the conserved Cys124 at the phosphatase domain changed to serine (c/s), was also inserted downstream of MMTV promotor. This construct inhibits both the lipid and protein phosphatase activity of MMAC1. In addition, we prepared a whey acidic protein (WAP) construct containing the c/s MMAC1 construct. The WAP promotor also targets to breast, but compared with MMTV regulates adequate gene expression during mid-pregnancy. Moreover, a Gly129Glu (g/e) phosphoinositide phosphotase-deficient MMAC1 was cloned into MMTV promotor. MMAC1 (g/e) lacks the ability to dephosphorylate lipids while leaving the protein phosphotase activity intact thus selectively acting as a dominant negative for the lipid phosphatase activity of MMAC1.

Task #2 To determine the effects of MMAC1 expression on mammary development and tumorigenesis.

We have obtained founders from each transgenic line: one founder for MMTV wt, three founders for MMTV c/s, one founder for WAP c/s and seven founders for MMTVG/E. Typical examples of positive mice in terms of expression assessed by cutting with BamH1 and HindII and southern blotting or PCR with primers unique to the transgene are presented in figure 1. To distinguish the heterozygous from non-transgenic mice, tail DNA was digested, separated on and agarose gel and hybridized with a ^{32}P-labeled MMAC1 cDNA probe. For a more sensitive approach, mouse tail DNA was amplified with PCR primers derived from MMAC1 cDNA and a transgene specific primer. The PCR products were examined on an agarose gel with the transgene status indicated by the band above the primer dimers. Expression of MMAC1 protein in the transgenic mice was determined by western blot with anti-MMAC1 antibody of breast tissue (Figure 1). The results indicated variable expression levels of the transgenes among the lines. Equal loading of samples was evaluated with blotting the membrane with anti-β-actin antibody.

Mice with relatively high expression of transgenes have been bred and followed for at least 6 months. We have followed 15 MMTV wt, 14 MMTV c/s, 35 MMTVg/e mice and 15 WAP c/s mice. None of the mice have yet developed palpable tumors. The mice will be followed for an

additional year. A similar number of mice will be bred multiple times to assess the effects of pregnancy and the resultant high level expression of the transgene on tumor development.

We have collected tissues from the mice to determine the effects of the transgene on breast development. These samples will be assessed with the assistance of Rhama Kohkhar a breast pathologist with experience in murine models. The mice will be bred to determine the effects on lactation and breast involution.

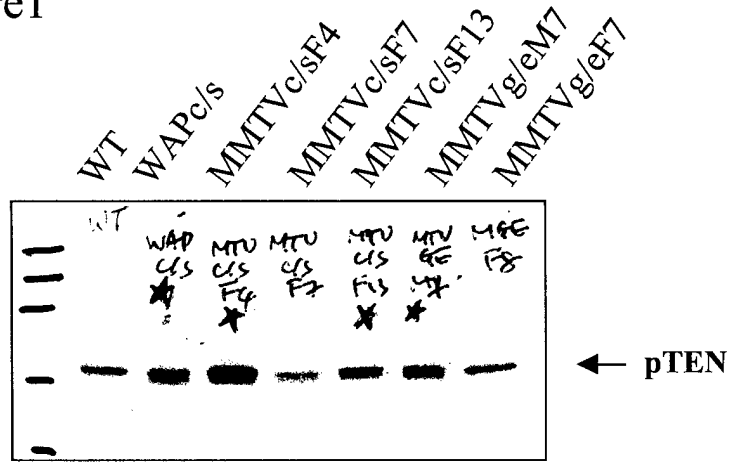
Task #3 To determine the effects of MMAC1 on breast tumorigenesis induced by other oncogenes and tumor suppressor genes.

To allow assessment of MMAC1 wt and dn constructs on tumor development, we need to cross the mice into a background where the appropriate transgenes or knockouts are available. We have crossed the lines with C57/Bl6 mice and are at the F4 generation.

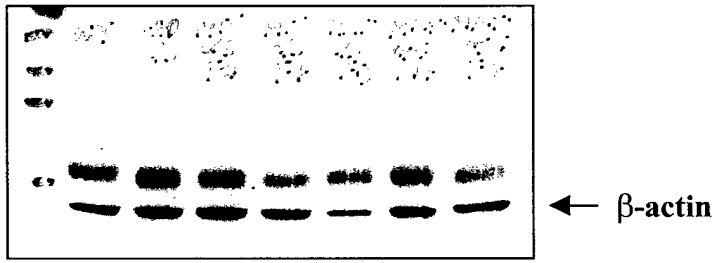
In the event that the MMAC1 transgenic mice do not produce primary tumors following the manipulations described above, we will cross thee mice with Wnt1 transgenic or p27 knock-out mice which are commercially available. Wnt1 and p27 have been shown to complement PTEN in tumor development in breast and prostate models. We will also assess the mice in a polyoma middle T system developed by Dr. W. Muller. Polyoma middle T is a potent tumor inducer. However, a mutant polyoma middle T which is incapable of linking to the phosphatidylinositol 3 kinase pathway does not induce tumors. We will assess whether activation or inhibition of MMAC1 function using the transgenes available will alter tumor development in the wild type and mutant polyoma system.

Figure1

A

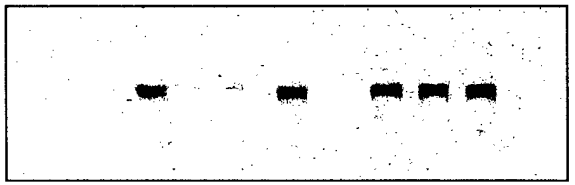


Blot : anti-pTEN



Blot : anti-actin

B



Mouse tail DNA were digested with BamHI + HindIII, run on agarose gel and hybridized with 32P pTEN cDNA probe.

C

Mouse tail DNA were amplified with PCR primers derived from pTEN cDNA .

Key Research Accomplishments

1). Generated trangene constructs for wild type MMAC1 under the MMTV promoter, a dominant negative (c/s) MMAC1 where the lipid and protein phosphatase activity is inhibited under the MMTV promoter, a dominant negative MMAC1 (g/e) where the lipid phosphatase activity is inhibited and the protein phosphatase is intact and a WAP MMAC1 dominant negative (c/s).

2) established transgenic mice and demonstrated breast specific expression of wild type MMAC1 under the MMTV promoter (1 founder), a dominant negative (c/s) MMAC1 where the lipid and protein phosphatase activity is inhibited under the MMTV promoter (3 founders) a dominant negative MMAC1 (g/e) where the lipid phosphatase activity is inhibited and the protein phosphatase is intact (7 founders), and a WAP MMAC1 dominant negative (1 founder).

3) followed each strain of mice with at least 15 progeny for 6 months to establish sensitivity to tumor development.

4) collected tissues to determine effect on breast development

5). Transferred each transgenic strain to the C57/Bl6 background by breeding and are at the F1-F4 generation.

Reportable Outcomes

None.

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